Interaction of 7-Bromoacetyl-7-desacetylforskolin, an Alkylating Derivative of Forskolin, with Bovine Brain Adenylyl Cyclase and Human Erythrocyte Glucose Transporter

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ABSTRACT: 7-Bromoacetyl-7-desacetylforskolin (BrAcFsk), an alkylating derivative of forskolin, activated adenylyl cyclase and irreversibly blocked high affinity forskolin binding sites in human platelet membranes and rat brain membranes (Laurenza et al., 1990). Photoincorporation of an iodinated arylazido derivative of forskolin, ^{125}I-6-AIPP-Fsk, into adenylyl cyclase in bovine brain membranes was irreversibly inhibited by BrAcFsk but not by 1,9-dideoxy-BrAcFsk, suggesting that BrAcFsk was reacting specifically with a nucleophilic group(s) at the forskolin binding site of adenylyl cyclase. Immunoblotting with antiforskolin antiserum demonstrated that partially purified bovine brain adenylyl cyclase had incorporated BrAcFsk. The interaction of BrAcFsk with the glucose transporter in human erythrocyte membranes was examined in a similar manner. Photoincorporation of ^{125}I-7-AIPP-Fsk, an iodinated arylazido derivative of forskolin which is specific for the glucose transporter, into the glucose transporter was not irreversibly inhibited by BrAcFsk, suggesting that, in contrast to adenylyl cyclase, there is no reactive nucleophilic group at the forskolin binding site on the human erythrocyte glucose transporter. The immunoblotting procedure with antiforskolin antiserum confirmed that BrAcFsk was not covalently attached to human erythrocyte glucose transporter.

Forskolin, a naturally occurring plant diterpene, stimulates adenylyl cyclase directly and has been used extensively to increase cellular cAMP levels and to elicit cAMP-dependent physiological responses (Seamon & Daly, 1986). High affinity binding sites for forskolin have been described in rat brain and human platelet membranes, and these sites have structural requirements for forskolin analogs which are similar to those for activation of adenylyl cyclase (Seamon et al., 1984; Nelson & Seamon, 1986). The high affinity of forskolin for adenylyl cyclase has prompted the synthesis of a matrix-bound derivative of forskolin, which has been used to affinity purify the enzyme from several tissues (Pfeuffer et al., 1985a,b; Smigel, 1986; Krupinski et al., 1989) and from Sf9 cells expressing recombinant calmodulin-activated (type-I) adenylyl cyclase (Tang et al., 1991). Photoactivatable derivatives of forskolin have been synthesized and shown to label specifically the adenylyl cyclase catalytic subunit from rabbit myocardia (Pfeuffer & Pfeuffer, 1989), rat adipocytes (Ho et al., 1988), and bovine brains (Morris et al., 1991a).

More recently, forskolin has been shown to affect a number of membrane transport proteins, including the glucose transporter, the multidrug transporter (or P-glycoprotein), and ligand- and voltage-gated ion channel proteins through a mechanism that does not involve the production of cAMP [see Laurenza et al. (1989) for review]. The direct interaction of forskolin with the glucose transporter has been demonstrated by binding of [^{3}H]forskolin to human erythrocytes (Lavis et al., 1987) and by the ability of both forskolin and 1,9-dideoxyforskolin, a naturally occurring analog of forskolin which does not activate adenylyl cyclase, to inhibit glucose transport and [^{3}H]cytochalasin B binding (Joost et al., 1988). Furthermore, [^{3}H]forskolin and ^{125}I-labeled azido derivatives of forskolin ([^{125}I]IAPS-Fsk,[1] [^{125}I]IASA-Fsk, and ^{125}I-7-AIPP-Fsk) have been photoincorporated into the erythrocyte glucose transporter (Shanahan et al., 1987; Wadzinski et al., 1987; Pfeuffer & Pfeuffer, 1989; Morris et al., 1991a). Forskolin and 1,9-dideoxyforskolin enhance the cytotoxic effects of adriamycin in a human ovarian carcinoma cell line, SKVLB, which overexpresses the P-glycoprotein and has the multidrug resistance (MDR) phenotype (Morris et al., 1991b). The direct interaction of forskolin with the overexpressed P-glycoprotein in this cell line has also been demonstrated by photoincorporation of ^{125}I-6-AIPP-Fsk and ^{125}I-7-AIPP-Fsk (Morris et al., 1991b).

The different membrane proteins affected by forskolin are predicted to have topologies in the membrane which are similar to that reported for adenylyl cyclase (Krupinski et al., 1989). The common motif is one or more regions consisting of six transmembrane segments being separated by cytoplasmic

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[1] Abbreviations: [^{125}I]IAPS-Fsk, (3-iodo-4-azidophenethyl)amido-7-*O*-succinyldeacetylforskolin; [^{125}I]IASA-Fsk, ((3-iodo-4-azidosalicylamido)ethyl)amido-7-succinyldeacetylforskolin; BrAcFsk, 7-bromoacetyl-7-desacetylforskolin; 1,9-dideoxy-BrAcFsk, 7-bromoacetyl-7-desacetyl-1,9-dideoxyforskolin; GTP-γ-S, guanosine 5′-*O*-(3-thio)triphosphate; ^{125}I-6-AIPP-Fsk, [^{125}I]-2-[3-(4-azido-3-iodophenyl)propanamido]-*N*-ethyl-6-(aminocarbonyl)forskolin; ^{125}I-7-AIPP-Fsk, [^{125}I]-2-[3-(4-azido-3-iodophenyl)propanamido]-*N*-ethyl-7-(aminocarbonyl)-7-desacetylforskolin; 7-AEC-Fsk, 7-[[(2-aminoethyl)amino]carbonyl]-7-desacetylforskolin; 6-AEC-Fsk, 6-[[(2-aminoethyl)amino]carbonyl]-forskolin; BSA, bovine serum albumin; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; CAPS, cyclohexylaminopropanesulfonic acid; WGA, wheat germ agglutinin; DTT, dithiothreitol; PBS, phosphate-buffered saline; Tris, Tris(hydroxymethyl)aminomethane; TBS, Tris-buffered saline; NEM, *N*-ethylmaleimide.

domains. These similarities make it tempting to speculate that forskolin may be binding at structurally similar sites on the proteins. However, many questions remain about these transmembrane glycoproteins, such as the definitive location of functional domains (e.g., the location of binding sites for D-glucose, cytochalasin B, and forskolin on the glucose transporter and the location of the binding domain(s) for forskolin on adenylyl cyclase). Structure–function studies with various analogs of forskolin demonstrated that the structural aspects of forskolin which are important for binding to adenylyl cyclase and the glucose transporter are different (Joost et al., 1988; Morris et al., 1991a; Robbins et al., 1991). The forskolin binding site on the glucose transporter has a higher affinity for lipophilic analogs of forskolin than the forskolin binding site on adenylyl cyclase (Joost et al., 1988; Wadzinski et al., 1987). Neither cytochalasin B nor D-glucose, both of which act at the glucose transporter, has any effect on high affinity [^{3}H]forskolin binding to the adenylyl cyclase or on the activity of adenylyl cyclase measured in the presence of forskolin (Joost et al., 1988). 1,9-Dideoxyforskolin inhibits both glucose transport and cytochalasin B binding, but it does not inhibit high affinity [^{3}H]forskolin binding to the adenylyl cyclase or activate adenylyl cyclase (Joost et al., 1988).

α-Haloacetyl analogs of forskolin were effective in activating adenylyl cyclase and irreversibly blocking forskolin binding sites in human platelet membranes and rat brain membranes (Laurenza et al., 1990) and in bovine brain membranes (Robbins et al., 1991). We used BrAcFsk, an alkylating derivative of forskolin, to begin to compare the structural features of the forskolin binding sites on bovine brain adenylyl cyclase and the human erythrocyte glucose transporter.

EXPERIMENTAL PROCEDURES

Materials

Lubrol-PX was from Pierce Chemical Co.; GTP-γ-S, pepstatin A, and leupeptin were from Boehringer Mannheim; [α-^{32}P]ATP, [^{3}H]cAMP, [γ-^{32}P]ATP, and ^{125}I-labeled protein A were from New England Nuclear; high molecular weight standards were from Bio-Rad; ^{14}C-labeled molecular weight standards were from Bethesda Research Laboratories; biotinylated wheat germ agglutinin (WGA) and Vectastain ABC-AP and substrate kits were from Vector Laboratories; and Immobilon-P was from Millipore. Forskolin–BSA conjugate and forskolin–KLH conjugate were prepared by reacting BSA or KLH with the *N*-hydroxysuccinimide ester of 7-desacetyl-7-hemisuccinylforskolin. BrAcFsk and 1,9-dideoxy-BrAcFsk were synthesized as described (Laurenza et al., 1990). 6-(Aminoethyl)carbamylforskolin (6-AEC-Fsk), (7-aminoethyl)carbamyl-7-desacetylforskolin (7-AEC-Fsk), and an isothiocyanate derivative of 6-AEC-Fsk were synthesized as described (Robbins et al., 1991). The forskolin affinity gel was prepared by coupling 7-AEC-Fsk to Affi-Gel 15 as described (Moos et al., 1990). The photoaffinity labels, ^{125}I-6-AIPP-Fsk and ^{125}I-7-AIPP-Fsk, were prepared as described (Morris et al., 1991a). The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart (Beavo et al., 1974) and provided by Dr. Sandra Rossie, Purdue University, West Lafayette, IN. Protein A Sepharose CL-4B was from Pharmacia LKB. Filter inserts (Ultrafree-MC Durapore, 0.45 μm) were from Millipore.

Methods

Isolation of Membranes. Crude membranes were isolated from bovine brains as described previously (Morris et al., 1990). Crude bovine brain membranes were preactivated by incubation with 10 mM $MgCl_2$ and 100 μM GTP-γ-S for 30 min at 30 °C as described (Morris et al., 1990) to form the stable complex of Gsα protein and catalytic subunit. Washed human erythrocyte membrane ghosts were prepared as described by Steck and Kant (1974).

Photoaffinity Labeling Procedure. The procedure was essentially the same as described previously (Morris et al., 1991a). ^{125}I-6-AIPP-Fsk (3 nM) and ^{125}I-7-AIPP-Fsk (3 nM) were incubated with preactivated bovive brain membranes and with human erythrocyte membrane ghosts, respectively, in a volume of 100 μL of buffer solution (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) for 30 min on ice in the dark. Immediately before photolysis, 1 mL of buffer solution was added and the diluted samples were quickly photolyzed (energy = 200 000 μJ) in microfuge tubes in a Stratagene UV Stratalinker. Following photolysis, 1% β-mercaptoethanol was added to each tube as a scavenger for any long-lived species. The membranes were pelleted and either resuspended in electrophoresis sample buffer or solubilized and immunoprecipitated as described below.

Pretreatment with Forskolin Analogs. Preactivated bovine brain membranes or human erythrocyte membrane ghosts were incubated in 100 μL of buffer solution in the absence or presence of 20 μM forskolin or analog (BrAcFsk, 1,9-dideoxy-BrAcFsk, or the isothiocyanate derivative of 6-AEC-Fsk) for 30 min on ice. The bovine brain membranes were either incubated with photolabel and photolyzed as described above or washed twice with 10 mL of buffer, collected by centrifugation at 11 000 rpm in a Sorvall SS-34 rotor at 4 °C, washed once with 1 mL of buffer solution, and collected by centrifugation in an Eppendorf microfuge for 15 min at 4 °C. The human erythrocyte membranes were washed three times with 1 mL of buffer solution and collected by centrifugation in an Eppendorf microfuge for 15 min at 4 °C. The washed membrane pellets were resuspended in 100 μL of buffer solution for photolysis as above or for immunoprecipitation as below.

Purification and Chemical Affinity Labeling of Adenylyl Cyclase with BrAcFsk. Adenylyl cyclase was purified from solubilized preactivated bovine brain membranes essentially as described by Krupinski et al. (1989) with some modifications as follows: the purification was carried out with approximately 25 mL of a forskolin affinity gel prepared with 7-AEC-Fsk (Moos et al., 1990); the column was washed with buffer containing 100 μM 1,9-dideoxyforskolin prior to the elution of adenylyl cyclase in order to remove other forskolin binding proteins; 20 μM BrAcFsk was substituted for 200 μM forskolin in the elution buffer; and DTT (1 mM) and forskolin (100 μM) were added to each fraction collected during elution of adenylyl cyclase activity to inactivate excess unincorporated BrAcFsk and to stabilize the enzyme, respectively. Adenylyl cyclase assays were carried out in the presence of 0.1 mM ATP, 5 mM $MnCl_2$, 5 mM $MgCl_2$, and 100 μM forskolin as described (Morris et al., 1990). Fractions containing peak adenylyl cyclase activity were pooled, concentrated approximately 5-fold on an Amicon YM 30 membrane, and stored in aliquots at −80 °C. The specific activity of the concentrated adenylyl cyclase preparation was 0.13 μmol (min·mg)$^{-1}$.

Phosphorylation by cAMP-Dependent Protein Kinase. The reaction was carried out essentially as described by Costa and Catterall (1984). Phosphorylation buffer components (25 mM HEPES/Na, pH 7.4, 10 mM $MgCl_2$, and 5 mM EGTA) were added to an aliquot of concentrated, BrAcFsk-eluted, partially purified adenylyl cyclase (approximately 75 pmol/

min) in a final volume of 100 μL. Cyclic AMP-dependent protein kinase catalytic subunit (approximately 500 ng) and carrier-free [γ-^{32}P]ATP (5 μCi) were added, and the sample was incubated for 5 min at 37 °C. The reaction was stopped by adding 100 μL of cold EDTA (100 mM), pH 7.4. Carrier protein (carbonic anhydrase, 30 μg) was added, and proteins were precipitated with deoxycholate and trichloroacetic acid as described (Peterson, 1983). The pellets were washed with acetone and analyzed by SDS–PAGE and autoradiography as described below.

Production of Antiserum. Antiforskolin antiserum was raised in rabbits against forskolin which was conjugated at the 7-position to keyhole limpet hemocyanin (KLH). Antiglucose transporter antiserum (379) was raised against a synthetic peptide corresponding to the C-terminal 13 amino acids of rat GLUT1 (Mueckler et al., 1985; Maher et al., 1991).

Immunoprecipitation of Glucose Transporter. Photolabeled human erythrocyte membranes (approximately 100 μg) were incubated in solubilization buffer (PBS plus 2% Thesit, 2 mM NEM, 5 mM EDTA, and 10 μg/mL each of phenylmethanesulfonyl fluoride, aprotinin, and pepstatin A) for 30 min on ice with periodic mixing and added to tubes containing antiglucose transporter antiserum (379) which has been prebound to protein A–Sepharose (by mixing 10 μL of antiserum with 100 μL of protein A–Sepharose (50% (v/v) slurry) for 2–4 h at 4 °C). The mixture was incubated overnight at 4 °C with rocking and the protein A–Sepharose was collected and washed twice with 1 mL of PBS plus 5 mM EDTA and 0.2% Thesit and once with 1 mL of PBS. The proteins were eluted from the protein A–Sepharose by incubation for 30 min at room temperature in Laemmli electrophoresis sample buffer containing urea (2.3 M) and DTT (50 mM). The Sepharose was removed by centrifuging the sample through Milipore 0.45 μm filter inserts into microfuge tubes for 5 min at 14 000 rpm in a microfuge.

SDS–PAGE. Photolabeled bovine brain membranes were incubated for 10 min at 60 °C in Laemmli electrophoresis sample buffer (Laemmli, 1970) which was modified to contain 1% SDS. Aliquots of partially purified adenylyl cyclase were precipitated with deoxycholate and TCA as described (Peterson, 1983) washed with acetone, and incubated for 10 min at 60 °C in Laemmli electrophoresis sample buffer (Laemmli, 1970) which was mofified to contain 20 mM β-mercaptoethanol. Iodoacetamide (80 mM) was added and samples were incubated for another 10 min at 60 °C. The high molecular mass standards were myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa). The ^{14}C-labeled molecular weight standards were myosin, phosphorylase B, bovine serum albumin, ovalbumin, and carbonic anhydrase (29 kDa). Electrophoresis of samples containing adenylyl cyclase was performed on minigels (7.5% or 8% acrylamide) in a Bio-Rad Miniprotean II electrophoresis apparatus using the discontinuous buffer system described by Laemmli (Laemmli, 1970) except SDS was omitted from the gels. Electrophoresis of samples containing glucose transporter was performed on 1.5-mm gels (10% acrylamide) using the discontinuous Laemmli buffer system (Laemmli, 1970). Gels were either transferred to Immobilon-P or nitrocellulose as described below, silver stained as described by Hochstrasser et al. (1988), or Coomassie-stained, dried, and exposed to Kodak XAR 5 film.

Electrophoretic Transfer and Immunodetection Procedure. Samples containing adenylyl cyclase were transferred from the gel onto Immobilon-P in 25 mM Tris, 10 mM glycine, 1% methanol for 30 min at 50 V, while samples containing glucose transporter were transferred from the gel onto nitrocellulose in 25 mM Tris, 192 mM glycine, 20% methanol for 12 h at 300 mA. For immunodetection with antiforskolin antiserum, the blot was incubated for 1 h at room temperature in TBS (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl) containing 5% BSA and incubated overnight at 4 °C in antiforskolin antiserum diluted 1:50 in TBS with 5% BSA. The blot was washed twice (5 min/wash) in TBS with 0.05% Tween 20 and twice with TBS and incubated 1 h at room temperature with ^{125}I-labeled protein A (0.2 μCi/mL) in TBS with 5% BSA. The blot was washed twice in TBS with 0.05% Tween 20, twice with TBS, air-dried, and exposed to Kodak XAR 5 film. Immunoblotting with H antiserum, an antihuman erythrocyte glucose transporter (GLUT1) antiserum [see Wheeler et al. (1982)], was performed at a dilution of 1:300 as described (Maher et al., 1991).

Biotinylated Wheat Germ Agglutinin (WGA) Binding Procedure. Proteins were separated by SDS–PAGE and transferred onto Immobilon as described above; the blot was incubated in buffer A (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20, and 1 mM $CaCl_2$) containing 3% gelatin and 0.1% BSA for 1 h at room temperature and then overnight at 4 °C. The blot was incubated for 2 h at room temperature in buffer A with 1% gelatin, 0.1% BSA, and biotinylated WGA (10 μg/mL) and washed three times in TTBS (100 mM Tris, pH 7.5 with 0.5 M NaCl and 0.1% Tween 20). The blot was incubated for 30 min in biotinylated alkaline phosphatase–avidin complex (ABC-AP) reagent and washed three times in TTBS. The blot was transferred to freshly prepared substrate solution until stained and finally washed in water.

RESULTS

Irreversible Inhibition of Forskolin Photoaffinity Labeling of Adenylyl Cyclase by BrAcFsk. The ^{125}I-labeled arylazido derivative of forskolin, ^{125}I-6-AIPP-Fsk, is specifically incorporated into the forskolin binding site of adenylyl cyclase in crude bovine brain membranes (Morris et al., 1991a). The specificity of the interaction of BrAcFsk with adenylyl cyclase was examined by determining whether forskolin, BrAcFsk, or 1,9-dideoxy-BrAcFsk would inhibit photoincorporation of ^{125}I-6-AIPP-Fsk into adenylyl cyclase in preactivated bovine brain membranes (Figure 1). Forskolin and BrAcFsk (at concentrations of 20 μM) inhibited photolabeling of adenylyl cyclase (M_r = 115 000) by ^{125}I-6-AIPP-Fsk (lanes 2 and 3, respectively). In contrast, 1,9-dideoxy-BrAcFsk, which does not activate adenylyl cyclase, did not inhibit photolabeling of adenylyl cyclase (lane 4).

To examine whether the binding of BrAcFsk at the forskolin binding site was irreversible, preactivated membranes were incubated with forskolin or analog and were washed before photolabeling to remove noncovalently bound forskolin or analog. In the case of membranes pretreated with forskolin (lane 6), the amount of photolabel incorporated after the membranes were washed was the same as in the control sample (lane 5), indicating that forskolin was reversibly bound and was efficiently removed by the washing procedure. In contrast, photolabeling of adenylyl cyclase following pretreatment with BrAcFsk was inhibited even after several washes of the membranes (lane 7), indicating that BrAcFsk bound irreversibly to adenylyl cyclase. 1,9-Dideoxy-BrAcFsk had no inhibitory effect on photolabeling after the membranes were washed (lane 8).

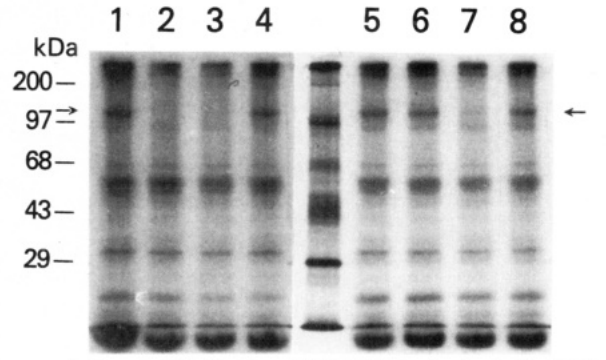

FIGURE 1: Irreversible binding of BrAcFsk to the forskolin binding site on adenylyl cyclase. Bovine brain membranes were preactivated with GTP-γ-S and treated as described in Methods with either no addition (lanes 1 and 5), 20 μM forskolin (lanes 2 and 6), 20 μM BrAcFsk (lanes 3 and 7), or 20 μM 1,9-dideoxyBrAcFsk (lanes 4 and 8). After the treatments, the membranes were either not washed (lanes 1–4) or washed (lanes 5–8) to remove noncovalently bound compound. The membranes were photolyzed in the presence of ^{125}I-6-AIPP-Fsk as described in Methods and subjected to SDS–PAGE (8% gel) and autoradiography. The arrows point to photolabeled adenylyl cyclase (M_r = 115 000). ^{14}C-Labeled molecular weight standards were run in the lane in between lanes 4 and 5.

The interaction of another alkylating forskolin derivative, the isothiocyanate derivative of 6-AEC-Fsk, which would also react with a nucleophilic group at the forskolin binding site was examined. The 6-isothiocyanate (at 20 μM) inhibited photolabeling of adenylyl cyclase in bovine brain membranes. Photolabeling of adenylyl cyclase following pretreatment with the 6-isothiocyanate derivative of 6-AEC-Fsk was inhibited even after several washes of the membranes, indicating that the 6-isothiocyanate bound irreversibly to adenylyl cyclase (data not shown).

Immunodetection of BrAcFsk-Labeled Proteins. A procedure was developed to detect the incorporation of BrAcFsk into proteins, which involved electrophoretic separation and transfer of BrAcFsk-labeled proteins onto Immobilon-P followed by detection with antiforskolin antiserum and ^{125}I-labeled protein A. The specificity of this detection method is demonstrated in Figure 2. Antiforskolin antiserum recognized a forskolin–BSA conjugate (lane 1), while no immunoreactivity was observed with unconjugated BSA (data not shown). The immunoreactivity was not inhibited by BSA which was included in the antibody buffer. Addition of KLH, the carrier protein to which the forskolin was conjugated to prepare the antiserum, did not inhibit detection (lane 2). However, the immunoreactivity was completely inhibited by 100 μM forskolin (lane 3) demonstrating that the antiserum was specific for forskolin.

Chemical Affinity Labeling of Bovine Brain Adenylyl Cyclase. BrAcFsk has been demonstrated to activate adenylyl cyclase in membrane preparations (Laurenza et al., 1990). Prior to studying the incorporation of BrAcFsk into solubilized adenylyl cyclase, we tested its ability to activate solubilized adenylyl cyclase which was extracted from bovine brain membranes with Lubrol. Forskolin activated solubilized adenylyl cyclase with an EC_{50} of approximately 1 μM. The same level of activation required 5–10 μM BrAcFsk. The maximum level of activation achieved with forskolin was 5-fold, whereas that achieved with BrAcFsk was 3-fold. These results were similar to those previously observed with human platelet membranes and rat brain membranes (Laurenza et al., 1990).

The Lubrol-solubilized extract from GTP-γ-S-preactivated membranes was incubated with 7-AEC-forskolin affinity gel; the gel was washed, and bound proteins were eluted with 20 μM BrAcFsk as described in Experimental Procedures. The eluted proteins were separated by SDS–PAGE, and those which had incorporated BrAcFsk were identified by the immunoblotting procedure described above (Figure 3). The silver-stained SDS–polyacrylamide gel (lane 1) showed that several proteins were present in the adenylyl cyclase preparation, including a diffuse band at 120 kDa which, on the basis of its apparent molecular weight, was presumed to be adenylyl cyclase. The blot developed with antiforskolin antiserum (lane 2) showed a predominant diffuse band at 120 kDa and a sharp band at 150 kDa. The intensity of the staining of the 120-kDa protein relative to the other proteins present in the sample was greater in the immunoblot than in the silver stained gel, suggesting that it was selectively labeled with BrAcFsk. No immunoreactivity was observed when the Western blot was developed with nonimmune rabbit serum (data not shown).

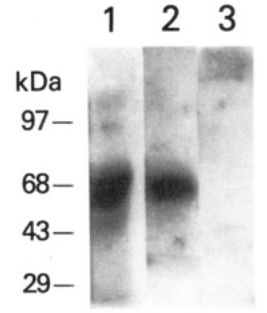

FIGURE 2: Specificity of antiforskolin antiserum in immunoblotting procedure. Aliquots of a forskolin–BSA conjugate (0.2 μg) were run on an 8% SDS–polyacrylamide gel and blotted onto Immobilon. The blots were incubated with antiforskolin antiserum (lane 1) or antiforskolin antiserum in the presence of either 1 mg/mL KLH (lane 2) or 100 μM forskolin (lane 3). The antibodies bound to the blots were detected by incubation with ^{125}I-labeled protein A as described in Methods.

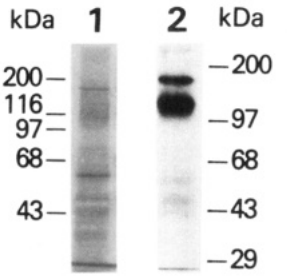

FIGURE 3: Chemical affinity labeling of partially purified adenylyl cyclase with BrAcFsk. Adenylyl cyclase was purified from bovine brain membranes by forskolin affinity chromatography with elution by BrAcFsk (20 μM) as described in Methods. Aliquots of the purified preparation (10–20 μg) were resolved by SDS–PAGE on 7.5% gels and the gels were processed as described in Methods. In lane 1, the gel was silver stained to detect total proteins. In lane 2, the proteins were electrotransferred to Immobilon and the Immobilon was incubated with antiforskolin antiserum followed by ^{125}I-labeled protein A.

To characterize the BrAcFsk-labeled 120-kDa protein present in the partially purified adenylyl cyclase preparation, we determined whether it had two structural features which were predicted from the deduced amino acid sequence of bovine brain adenylyl cyclase (Krupinski et al., 1989). A consensus site for phosphorylation by cAMP-dependent protein kinase is present in the deduced amino acid sequence as well as four possible N-linked glycosylation sites, only one of which is modeled as being exposed extracellularly (Krupinski et al., 1989). The BrAcFsk-labeled, partially purified preparation was subjected to in vitro phosphorylation by incubation with cAMP-dependent protein kinase catalytic subunit and [γ-^{32}P]-

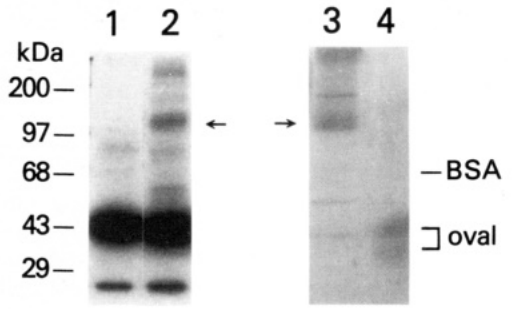

FIGURE 4: Characterization of proteins in the BrAcFsk-eluted, partially purified adenylyl cyclase preparation. Cyclic AMP-dependent protein kinase catalytic subunit and [γ-^{32}P]ATP were incubated (as described in Methods) in the absence (lane 1) or presence (lane 2) of the partially purified preparation of adenylyl cyclase which had been eluted with BrAcFsk. The samples were analyzed by SDS–PAGE on a 7.5% gel and autoradiography. The arrows indicate the mobility of the 120-kDa protein. The partially purified adenylyl cyclase preparation which had been eluted with BrAcFsk (lane 3) and a mixture of ovalbumin and BSA (lane 4) were subjected to SDS–PAGE on a 7.5% gel, electrophoretically transferred to Immobilon, and detected with biotinylated WGA and alkaline phosphatase-coupled avidin–biotin complex. Ovalbumin (labeled oval) appeared as a doublet. The expected position of bovine serum albumin (labeled BSA) is marked.

ATP as previously described (Costa & Catterall, 1984). Analysis by SDS–PAGE and autoradiography (Figure 4) showed that the 120-kDa protein was phosphorylated in the presence of cAMP-dependent protein kinase catalytic subunit (lane 2). The lower molecular weight heavily phosphorylated protein is the protein kinase catalytic subunit which underwent autophosphorylation during the reaction. Only this band was evident in the control sample which did not contain the partially purified adenylyl cyclase preparation (lane 1). The 120-kDa protein bound biotinylated WGA (Figure 4, lane 3) and, therefore, must contain at least one oligosaccharide containing either *N*-acetylglucosamine or sialic acid. This staining procedure was specific for glycosylated proteins (see lane 4) since ovalbumin (M_r = 43 000), which contains N-linked oligosaccharides, bound biotinylated WGA, while BSA (M_r = 68 000), which is not a glycoprotein, did not bind biotinylated WGA. Although they are not proof, these results are consistent with the 120-kDa protein in the BrAcFsk-eluted, partially purified preparation being adenylyl cyclase.

Interaction of BrAcFsk with the Glucose Transporter in Human Erythrocyte Membranes. ^{125}I-7-AIPP-Fsk has been shown to photolabel the glucose transporter of human erythrocyte membranes specifically and the photolabeling was inhibited in the presence of BrAcFsk (Morris et al., 1991a). The interaction of BrAcFsk with the glucose transporter was examined further by determining whether BrAcFsk would irreversibly inhibit photoaffinity labeling with ^{125}I-7-AIPP-Fsk. Erythrocyte membranes were pretreated with either forskolin or BrAcFsk at a concentration of 100 μM and washed to remove noncovalently bound forskolin or analog. Following this pretreatment, the membranes were photolyzed in the presence of ^{125}I-7-AIPP-Fsk, solubilized, and immunoprecipitated with antiglucose transporter antiserum. The immunoprecipitated proteins were separated by SDS-PAGE (Figure 5A). The glucose transporter immunoprecipitated from photolabeled control membranes appeared as a broad band (M_r = 43 000–70 000) which was heavily labeled with ^{125}I-7-AIPP-Fsk (lane 1). After pretreatment with forskolin (lane 2) or BrAcFsk (lane 3) followed by washing, the glucose transporter was photolabeled to the same extent as the control sample, indicating that the binding of forskolin and BrAcFsk was reversible.

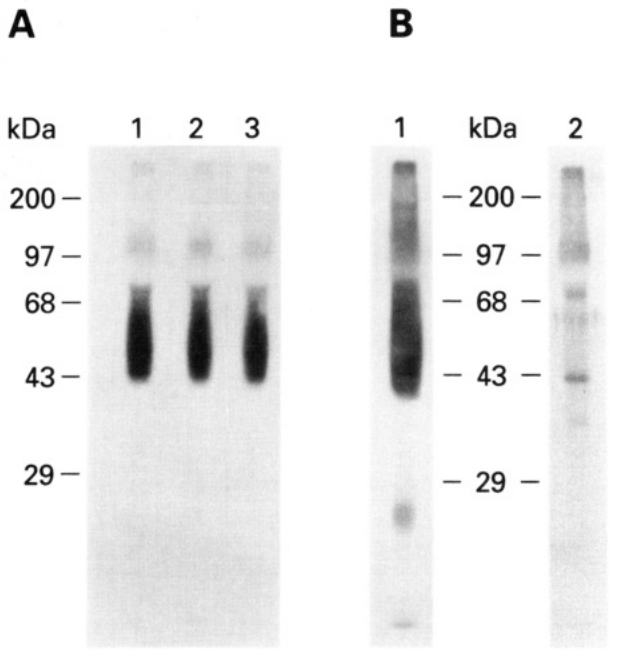

FIGURE 5: Reversible binding of BrAcFsk to the glucose transporter. (A) Human erythrocyte membranes were treated with either no addition (lane 1), 20 μM forskolin (lane 2), or 20 μM BrAcFsk (lane 3) and washed to remove noncovalently bound forskolin or analog. Following this pretreatment, the membranes were photolyzed in the presence of ^{125}I-7-AIPP-Fsk, washed, solubilized, and immunoprecipitated with antiglucose transporter antiserum as described in Methods. The immunoprecipitated proteins were subjected to SDS–PAGE on a 10% gel and autoradiography. (B) Human erythrocyte membranes were reacted with BrAcFsk (20 μM), washed to remove noncovalently bound analog, and immunoprecipitated with antiglucose transporter antiserum. The immunoprecipitated proteins were divided in half, separated by SDS–PAGE on a 10% gel, and immunoblotted with antiglucose transporter antiserum (lane 1) and antiforskolin antiserum (lane 2).

The interaction of BrAcFsk with the erythrocyte glucose transporter was also examined by the immunoblotting technique with antiforskolin antiserum. Human erythrocyte membranes were incubated with BrAcFsk (100 μM) and washed to remove BrAcFsk which was not covalently bound, and the glucose transporter was immunoprecipitated with antiglucose transporter antiserum (Figure 5B). The sample was divided in half and the proteins in each half were separated by SDS–PAGE and electrophoretically transferred onto nitrocellulose. The blots were incubated with either antiglucose transporter antiserum or antiforskolin antiserum followed by ^{125}I-labeled protein A. The blotted immunoprecipitate was immunoreactive with antiglucose transporter antiserum (lane 1). In contrast, no incorporation of BrAcFsk into the erythrocyte glucose transporter was detected by immunoblot analysis with antiforskolin antiserum (lane 2). The characteristic very broad band associated with the glucose transporter was not apparent in lane 2. The other proteins detected with antiforskolin antiserum in the immunoprecipitate from erythrocyte membranes (lane 2) were heavily labeled with BrAcFsk in crude membrane preparations and were observed when a sample was immunoprecipitated with normal rabbit serum and processed in the same manner (data not shown). The 43-kDa BrAcFsk-labeled protein in lane 2 may be actin, a major erythrocyte membrane protein which has high affinity binding sites for cytochalasin B (Flanagan & Lin, 1980; Shanahan, 1983) and binds ^{125}I-7-AIPP-Fsk and forskolin affinity columns.[2]

[2] M. Moos, Jr., D. I. Morris, and K. B. Seamon, unpublished observation.

DISCUSSION

Alkylating agents are useful reagents for determining whether ligand binding sites have reactive nucleophilic groups, such as cysteines or histidines. Binding sites that contain such nucleophilic groups may be alkylated and will be modified irreversibly, inhibiting the interaction of other agents. The advantage of using a chemically reactive reagent, such as an alkylating agent, is the ability to covalently modify a stoichiometric amount of binding sites. This is in contrast to the use of photoaffinity agents, which have relatively low insertion yields. The disadvantage of using alkylating agents is their long life, which can lead to nonspecific binding to other proteins containing reactive groups. This is not as much of a problem with photoaffinity agents, which are destroyed upon photolysis. The purpose of this study was to determine if an alkylating derivative of forskolin could be used to discriminate between the forskolin binding sites on bovine brain adenylyl cyclase and the human erythrocyte glucose transporter. This required developing techniques for measuring the irreversible modification of binding sites, as well as techniques for the direct measurement of proteins covalently modified by BrAcFsk.

Affinity Labeling of the Forskolin Binding Site on Bovine Brain Adenylyl Cyclase. To indirectly assess the specificity and irreversibility of the binding of BrAcFsk, we examined the effects of BrAcFsk on photoaffinity labeling of adenylyl cyclase. BrAcFsk irreversibly inhibited photolabeling of the forskolin binding site on adenylyl cyclase in bovine brain membranes. Similar results were obtained with the 6-isothiocyanate derivative of forskolin which also attacks nucleophilic groups. These results demonstrate that BrAcFsk and the 6-isothiocyanate derivative interact with adenylyl cyclase at a site which disrupts photoincorporation, presumably at or near the same high affinity binding site as ^{125}I-6-AIPP-Fsk. In contrast, 1,9-dideoxy-BrAcFsk did not inhibit photolabeling of adenylyl cyclase, suggesting that the inhibitory effect was not due to nonspecific alkylation.

The irreversible inhibition of photolabeling by BrAcFsk is not observed when BrAcFsk is prereacted with β-mercaptoethanol. BrAcFsk was reacted with β-mercaptoethanol and the resulting thioether derivative was purified and characterized by NMR and mass spectral analysis (J. D. Robbins, unpublished data). This derivative inhibited the binding of ^{125}I-6-IHPP-Fsk to adenylyl cyclase with a K_d of 230 ± 14 nM and inhibited the photolabeling of adenylyl cyclase with ^{125}I-6-AIPP-Fsk (data not shown). However, the inhibition of photolabeling was not irreversible. Therefore, the ability of BrAcFsk to irreversibly inhibit photolabeling requires a reactive alkylating group.

Proteins covalently modified with BrAcFsk could be detected by Western blotting with antiserum raised against forskolin. However, several proteins were detected in crude bovine brain membranes that had been pretreated with BrAcFsk (data not shown). Therefore, BrAcFsk was used to elute adenylyl cyclase from a forskolin affinity column in order to increase the specificity of the labeling. A 120-kDa protein was the major protein detected with antiforskolin antiserum in the material eluted from the forskolin affinity column. This result suggests that BrAcFsk was reacting directly with the nucleophilic group(s) on adenylyl cyclase. It is presumed that BrAcFsk is reacting at the forskolin binding site of adenylyl cyclase eluted from the forskolin column in the same way it reacted at the forskolin binding site of the cyclase in the brain membranes. This is consistent with studies showing that [^{3}H]-forskolin binding sites in rat brain membranes, solubilized calmodulin-sensitive adenylyl cyclase from rat brain, and particulate enzyme from platelets were sensitive to treatment with the sulfhydryl reactive agent, *N*-ethylmaleimide (Seamon et al., 1985; Awad et al., 1983).

Treatment of bovine brain membranes with activating ligands promoted a strong interaction between Gsα protein and adenylyl cyclase (Morris et al., 1990). The adenylyl cyclase–Gs$_\alpha$ protein complex was stable to membrane solubilization, forskolin affinity chromatography, and immunoprecipitation with an antibody raised against the carboxy-terminal decapeptide of the Gsα subunit (Morris et al., 1990). Therefore, in the experiments reported here on the partially purified adenylyl cyclase, it is likely that the Gsα protein was associated with the eluted adenylyl cyclase, since the brain membranes were preactivated with GTP-γ-S before the purification procedure. However, the Gsα protein was not labeled by BrAcFsk to a significant extent since little, if any, BrAcFsk was incorporated into low molecular weight proteins (Figure 3, lane 2). This result suggests that the irreversible loss of [^{3}H]forskolin binding sites which was previously observed in BrAcFsk-treated human platelets and rat brain membranes (Laurenza et al., 1990) was primarily due to covalent modification of adenylyl cyclase catalytic subunit.

The results reported here demonstrate that BrAcFsk irreversibly binds to a site on adenylyl cyclase which affects the interaction between cyclase and ^{125}I-6-AIPP-Fsk. The fact that BrAcFsk affects adenylyl cyclase activation by the Gs protein (Laurenza et al., 1990) and blocks the high affinity forskolin binding site is consistent with previously observed synergistic interactions between forskolin and activated Gsα. However, BrAcFsk did not result in an inhibition of forskolin stimulated adenylyl cyclase suggesting that the high affinity sites for forskolin binding and photolabeling might be different from the sites associated with stimulation of the enzyme. These results are difficult to interpret because of the possibility for multiple types of adenylyl cyclase. It is possible that the various subtypes of adenylyl cyclase which exist in bovine brain and have been shown to differ in their interaction with Gs protein subunits (Feinstein et al., 1991; Tang et al., 1991) may differ in their reversible and irreversible interactions with forskolin and BrAcFsk (depending on whether or not a reactive cysteine exists at or near the site). To begin to examine these possibilities, future experiments are aimed at using the techniques described in this paper to analyze the effects of forskolin and alkylating derivatives of forskolin on the different types of adenylyl cyclase expressed individually in a recombinant expression system.

A significant amount of BrAcFsk was also incorporated into a 150-kDa protein in the partially purified adenylyl cyclase preparation. The 150-kDa protein also bound biotinylated wheat germ agglutinin. Further experimentation is necessary to determine whether this 150-kDa protein is a calmodulin-insensitive 150-kDa form of brain adenylyl cyclase which was reported to bind to wheat germ agglutinin–Sepharose (Mollner & Pfeuffer, 1988) or some other forskolin binding protein, such as clathrin, which is present in preparations of adenylyl cyclase purified by forskolin affinity chromatography.[3]

Interaction of BrAcFsk with the Human Erythrocyte Glucose Transporter. BrAcFsk inhibited photolabeling of the glucose transporter by ^{125}I-7-AIPP-Fsk, suggesting that BrAcFsk and the photolabel were binding at or near the same site (Morris et al., 1991a). However, pretreatment of erythrocyte membranes with BrAcFsk did not lead to

[3] M. Moos, Jr., and K. B. Seamon, unpublished observation.

irreversible inhibition of ^{125}I-7-AIPP-Fsk labeling of the glucose transporter (Figure 5). This was verified by the lack of immunoreactivity of the immunoprecipitated glucose transporter to antiforskolin antiserum after pretreatment with BrAcFsk. These results suggest that in contrast to adenylyl cyclase, there is no reactive nucleophilic group at the forskolin binding site on the erythrocyte glucose transporter. Studies have shown that there is at least one reactive exofacial sulfhydryl on the human erythrocyte glucose transporter and alkylation of this sulfhydryl by glutathione maleimide inhibits hexose transport and cytochalasin B binding (May, 1988). The inability of BrAcFsk to bind irreversibly to the glucose transporter suggests that BrAcFsk does not react with this reactive thiol group.

It has been suggested that forskolin may be binding at a structurally similar site on several membrane transport proteins with similar membrane topologies (Laurenza et al., 1989). The position of covalent attachment of [^{125}I]IAPS-forskolin into the glucose transporter has been localized to the tenth transmembrane helix (Wadzinski et al., 1990). At least part of the ^{125}I-6-AIPP-Fsk labeling site was associated with the C-terminal half of the P-glycoprotein (Morris et al., 1991b). However, the forskolin binding site on the P-glycoprotein may consist of membrane-spanning domains coming from both the N- and C-terminal halves, as is the case for other drug-binding domains in the P-glycoprotein (Bruggemann et al., 1989; Greenberger et al., 1991). It will be interesting to determine where the cysteine or histidine residue which reacts with BrAcFsk is localized on adenylyl cyclase and whether or not this residue is absent at a similar site in the glucose transporter.

Conclusions. An immunodetection procedure has been developed to identify proteins which react covalently with BrAcFsk, an alkylating derivative of forskolin. When purified proteins were treated with BrAcFsk, antiforskolin antiserum specifically recognized the BrAcFsk moiety covalently bound to proteins on immunoblots. These studies suggest the presence of a nucleophilic group at the forskolin binding site on bovine brain adenylyl cyclase and the absence of such a group on the human erythrocyte glucose transporter. The techniques used in this study may be useful for identifying whether reactive nucleophilic groups exist in forskolin binding sites on other types of adenylyl cyclase, on other types of glucose transporter, and on other forskolin binding proteins.

Alkylating reagents, such as BrAcFsk, and photoaffinity labels, such as ^{125}I-6- and ^{125}I-7-AIPP-Fsk, have different technical advantages and therefore may be used to complement one another. Whereas the affinity labels react covalently with nucleophiles (Wilcheck & Givol, 1977; Dickinson et al., 1985), the photoaffinity labels form nitrenes which insert into chemical bonds (Ruoho et al., 1984). Although chemical affinity labels may not be as specific as photoaffinity labels, they have higher rates of incorporation. The results reported here suggest that when enough purified adenylyl cyclase is obtained, the combination of chemical affinity labeling with BrAcFsk and monitoring incorporation with antiforskolin antiserum should be useful in conjunction with enzymatic digestion and amino acid sequence analysis to localize the forskolin binding site.

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SUPPLEMENTARY MATERIAL AVAILABLE

One figure showing the effects of BrAcFsk, 7-((β-hydroxyethyl)thio)-7-desacetylforskolin, and the isothiocyanate derivative of 6-AEC-Fsk on photolabeling of the forskolin binding site on adenylyl cyclase (1 page). Ordering information is given on any current masthead page.

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